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Expression of cell adhesion molecules and catecholamine synthesizing enzymes in the developing rat adrenal gland

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Cell adhesion molecules play a major role in determining tissue architecture during histogenesis. This immunocytochemical study of the adrenal gland examines the embryonic and early postnatal cellular expression of two neural cell adhesion molecules, NCAM and L1, which are widely expressed in brain and have been found also to be expressed in the adult rat adrenal gland. In parallel, antibodies directed against two neuroendocrine cell markers, tyrosine hydroxylase and phenylethanolamine *N*-methyltransferase, were employed to verify the phenotypic nature of developing chromaffin cells in order to correlate cell adhesion molecule expression with the state of chromaffin cell differentiation. NCAM was found to be expressed by chromoblasts within extra-adrenal blastema (i.e. before their migration into the cortical primordium) at the 16th day of embryonic life. It continued to be expressed by all developing chromaffin cells after their infiltration into the developing adrenal gland at all ages. L1 was also expressed by chromoblasts in extra-adrenal sites, but was found only in a subpopulation of chromaffin cells within the cortical primordium from the 16th embryonic day onwards. Those chromoblasts which expressed L1 constituted relatively large compact cell clusters within the gland at this stage, while intra-adrenal chromaffin cells not expressing L1 were dispersed in small cell groups. L1 was also strongly expressed by nerve fibres (and their surrounding Schwann cells) which appeared to innervate cell groups as early as the 16th embryonic day. Both extra- and intra-adrenal chromoblasts expressed tyrosine hydroxylase, but the large L1-positive cell aggregates were less intensely immunoreactive for tyrosine hydroxylase than were cells in small groups. PNMT expression was restricted to L1-negative intra-adrenal chromoblasts present in small groups. Ultrastructural observations demonstrated that cells expressing L1 contained few secretory granules at the 18th embryonic day. It is concluded from these data that these chromoblasts are the precursors of the noradrenergic cells found in the mature gland. In addition, the arrangement of noradrenergic chromaffin cells in the form of homotypic cell groups throughout the course of histogenesis of the adrenal medulla is likely to be a direct consequence of the exclusive co-expression of both NCAM and L1 by this subpopulation of maturing chromaffin cells.

INTRODUCTION

Adrenal medullary chromaffin cells originate from the same precursor cells in the neural crest as sympathetic ganglionic neurones and constitute with them and the small intensely fluorescent, SIF, cells the so-called sympathoadrenal cell lineage[5,33]. During migration in the primary sympathetic ganglionic chains, environmental influences have for many years been thought to determine the fate of these cells[15,38,39,45,60]. A proportion of cells migrating from the neural crest terminate in sympathetic ganglionic primordia, where they express a neuronal phenotype: those cells destined to express an endocrine phenotype in the adrenal gland continue their migration into the secondary sympathetic ganglionic chains and accumulate close to the adrenal cortical primordium, as a collection of extra-adrenal immature future chromaffin cells forming an "extra-adrenal blastema"[17,65] before infiltrating this tissue. Tyrosine hydroxylase, TH, the rate-limiting enzyme in the synthesis of catecholamines, has been reported to be present not only in such chromoblasts but to be expressed early during migration by both future sympathetic neurones and chromaffin cells[14,60]. More recently, various neuronal cell markers, including the protein gene product, PGP^{9} 5, have been found in early developing adrenal glands[31]. Characteristically neuronal mRNAs such as SCG-10, which are abundant in sympathetic ganglia, have also been found both in extra-adrenal chromoblasts and those already situated

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in the cortical primordia at embryonic day 15[6,7]: these appear to be down-regulated however during development. In addition, both the 68 kDa and 160 kDa neurofilament proteins, which are also expressed early in sympathetic ganglia[13], have been reported to be present at least in some of these chromoblasts[7,66]. In contrast, PNMT, the final enzyme in the synthesis of adrenaline characteristic of adrenergic chromaffin cells, has not been found in extra-adrenal chromoblasts but is induced only after cells have entered the cortical primordium[10,64,65].

A limited number of cell type specific membrane glycoproteins are considered to be responsable for establishing the architecture of tissues during development[23,24]. At the present time however, little or no data has been published on the cellular expression during adrenal gland development of two of these cell adhesion molecules (CAMs), NCAM and L1, which have in the past been considered to be characteristic of neurones (for reviews see refs. 26, 37, 46, 51).

NCAM represents a family of glycoproteins which are expressed widely during early embryogenesis[61]; after neural induction NCAM expression is largely, but not exclusively, limited to neural or neural crest derived tissues[11]. NCAM has been found in adult neuroendocrine tissues, including the adrenal medulla[34], although its polypeptide composition differs markedly from that of NCAM isolated from brain[35]. We have previously shown that NCAM is expressed by all chromaffin cells in the adult adrenal gland and suggested it may play a determinant role in early stages of histogenesis of this gland, in stabilising initial cell contacts[35]; its continued expression and synthesis in adult tissues however indicates that it may continue to be important in the maintenance of tissue structures.

In contrast, L1, a neural cell adhesion molecule first identified in the mouse[41] equivalent to the chick neuron-glia CAM[23,24] and NILE glycoprotein[9,47,54], is in general found to be expressed later during embryogenesis than NCAM[20]. It has a more restricted cellular expression (e.g. it appears to be absent from astroglial cells) and appears to be concentrated in particular cellular compartments, such as cell extensions[12,29,48,49,57,58]. The adult neuroendocrine tissue distribution of L1 differs markedly from that of NCAM[36]. In the adrenal medulla of adult rats, it is present in Schwann cells, but only a subpopulation of chromaffin cells, representing about 20% of all neuroendocrine cells in this tissue, express it[40]. In this previous study we showed that chromaffin cells which express L1 correspond to the noradrenergic cell population, while adrenergic chromaffin cells do not express L1.

Because of the fundamental biological functions ascribed to these molecules and their interest as "neuronal" cell markers, it is particularly important to investigate the cellular expression of NCAM and L1 during development of neuroendocrine tissues, when the choice between the future neuronal or neuroendocrine phenotype is determined, in order to understand the molecular mechanisms underlying the structural organisation of these tissues. Recent reports of cooperativity between NCAM and L1 in reinforcing adhesive interactions between cells[30] emphasizes the necessity of studying the expression of both in parallel. Moreover, in the developing adrenal gland it is possible to follow the state of chromaffin cell differentiation with the aid of marker enzymes of catecholamine metabolism, the developmental pattern of which have been well documented for this tissue[10,64,65]. Thus in the present study, possible correlations were sought between the expression of adhesion molecules, which may in part reflect certain neuronal traits, and the development of the adrenergic and noradrenergic phenotypes of maturing chromaffin cells.

MATERIALS AND METHODS

Tissue preparation

Embryonic age was defined as the number of days after the day of mating (E0). Embryos were removed from ether-anaesthetised pregnant rats. Anaesthetised embryonic or neonatal rats were either frozen immediately with liquid nitrogen cooled isopentane at −50°C or fixed at room temperature for 4 h in phosphate-buffered formaldehyde freshly prepared from *p*-formaldehyde (4%, 0.1 M phosphate buffer, pH 7.2). Fixed tissues were placed in 25% sucrose solutions and left overnight at 4°C before freezing. Cryostat sections of either intact embryos or isolated glands were cut at 10–12 μm, mounted on gelatin-chrome alum coated slides, air-dried and stored at −20°C until used.

Immunocytochemistry

Sections of tissues not previously fixed were fixed 30 min in formaldehyde before immunocytochemistry. Sections were immunolabelled by the indirect immunofluorescence method as previously described[40]. Briefly, slides were rehydrated in phosphate buffered saline (PBS) before incubation in normal sheep serum (5% in PBS, 30 min) to reduce non-specific background labelling. This was followed by incubation with primary antisera for 1–2 h, PBS washes, and incubation in fluorescein isothiocyanate or rhodamine-conjugated secondary antibodies for 1–2 h in the dark. Sections were viewed after extensive washing in PBS with a Zeiss microscope equipped with fluorescence optics and photographed on Ilford HP5 film rated at 800 ASA.

Antibodies directed against L1, NCAM, tyrosine hydroxylase (TH), and phenylethanolamine *N*-methyl transferase (PNMT) were employed as primary antibodies. Anti-L1 was a rabbit purified immunoglobulin fraction, generously supplied by Dr. F. Rathjen, Hamburg, Germany, and has been previously characterised[50]. Anti-NCAM was a rabbit polyclonal, a generous gift of Dr. C. Goridis, Marseille, France. Anti-TH, a mouse monoclonal was obtained from Euromedex, France. Two anti-PNMT sera were used. Rabbit anti-PNMT serum was a generous gift of Dr. L. Denoroy, Département de Médecine Expérimentale, CNRS, Université Claude Bernard, Lyon, France, and has been previously characterised on bovine chromaffin

cell cultures[44]. Sheep anti-PNMT serum was purchased from Euromedex, France. Anti-TH, anti-PNMT and anti-NCAM sera were diluted 1:250, 1:500 (for the rabbit serum) or 1:200 (for the sheep serum) and 1:2,000, respectively. L1 antibodies, dissolved in PBS at a concentration of 1.6 mg./ml., were diluted 1:600. Secondary antisera (Euromedex, France) were diluted 1:100. All antibodies were diluted in PBS containing 1% normal sheep serum, except for the sheep anti-PNMT serum.

Electron microscopy

Electron microscopy was employed to examine the morphological appearance of clusters of L1-immunoreactive cells in early adrenal glands. For this, 50-μm-thick vibratome sections of prefixed glands were immunolabelled for L1 by the indirect peroxidase method, using the same antibody dilution as for immunofluorescence, and processed for electron microscopy as previously described[35].

Morphometry

Cell counts were performed on cryostat sections of embryonic and early postnatal adrenal glands double immunolabelled for TH and L1. Intra-adrenal chromoblasts were classified into TH-positive, weakly TH-positive and L1-immunoreactive cells. L1-positive chromaffin cells were distinguished from L1-positive Schwann cells by phase contrast microscopy. Due to relatively wide variations in cell numbers depending on the incidence of the sections, all labelled cells were counted on each of several (at least six) sections for each age. Slides double-labelled for TH and NCAM revealed that all TH-positive cells (i.e. both strongly and weakly labelled) were also NCAM-positive.

RESULTS

16th embryonic day

At E16 the adrenal gland consists of the cortical primordium, to which is attached an extra-adrenal blastema composed of future chromaffin and satellite glial cells. Cells in this blastema expressed both NCAM and L1 (Fig. 1a,b). They were weakly immunolabelled for TH but were unreactive with antibodies to PNMT. Rabbit and sheep anti-PNMT sera gave identical results. The outermost layers of the cortical primordium were also labelled with antibodies against NCAM but were not stained with anti-L1 antibodies. At this stage, relatively few cells have migrated into the cortical primordium: those which have, were dispersed either in small groups separated by cortical cells or in larger compact cell clusters. The small groups of cells were NCAM-, TH- and also PNMT-positive, although immunofluorescence for the latter enzyme is relatively weak compared with that observed at later stages, but were not L1-positive. The larger clusters were both NCAM- and L1-positive. The L1-reactive chromoblasts within the gland were always found in such compact cell clusters, which were similar to those found more frequently at E18 (described in more detail below). Immunofluorescence for TH was less intense in these clusters than that of cells in small groups. Cells in large clusters were unreactive with anti-PNMT antibodies. The groups of L1-immunoreactive chromoblasts were relatively scarce compared with groups of NCAM-reactive cells at this stage. One or no groups of L1-positive cells per section were found compared with up to 10–15 groups of NCAM-positive cells. A striking feature of the L1 immunolabelling pattern at this stage was the intense fluorescence of nerve fibres and their associated Schwann cells coursing through the adrenal cortical primordium, apparently making contact with both large and small groups of chromoblasts. These nerve fibres were also NCAM-positive.

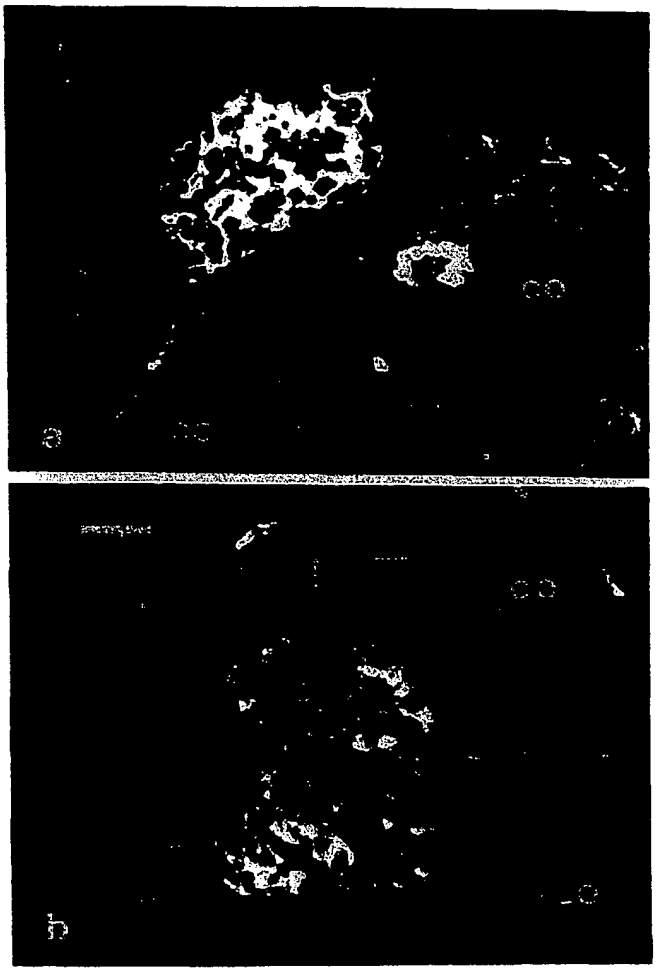

Fig. 1. Immunofluorescent staining of E16 extra-adrenal gland blastema for NCAM (a), and L1 (b). Many cells are stained for both adhesion molecules in the blastema. In addition the periphery of the cortical primordium (co) is stained for NCAM. Bar = 40 μm.

18th embryonic day

The staining pattern of cells constituting the extra-adrenal blastema was similar to that observed at E16 with regard to both the cell adhesion molecules and the two catecholaminergic metabolic enzymes examined. Chromoblasts within the cortical primordium were found, as at E16, to be dispersed either in relatively large compact cell clusters or in smaller groups. Large clusters measured up to 150 μm in diameter and contained up to 100–200 cells per section, while small

groups contained between 2 and 20 cells per section. The ratio of the number of small groups to large cell clusters of chromoblasts per section was greater than 15:1. Cells in large clusters were smaller and rounder with less cytoplasm than those in small groups, which generally had short blunt processes, but both were intensely reactive for NCAM (Fig. 2a,b). In contrast, TH immunofluorescence was consistently more intense in cells of small groups compared with that of the majority of cells forming the large clusters (Fig. 2c,d). The bulk of cells in large clusters reacted weakly with anti-TH antibodies, but the most peripheral cells in the large clusters were frequently as intensely immunofluorescent as those in small groups (Figs. 2d, 3a). As at E16, L1-positive chromoblasts within the gland were found at this stage only in the large compact cell clusters. Double labelling sections for TH and L1 demonstrated that the cell clusters that were positive for L1 were those that were less intensely reactive with anti-TH antibodies (Fig. 3a,b), while cells intensely fluorescent for TH were L1-negative (Fig. 3c,d). Chromoblasts reactive with anti-PNMT antibodies were found dispersed essentially in small cell groups. Double labelling of sections for TH and PNMT showed that the large cell clusters that were less intensely reactive with anti-TH antibodies were negative or reacted very weakly for PNMT (Fig. 3e,f) except for small clumps of cells occasionally attached to the periphery of such cell clusters, which were also those cells more reactive with anti-TH antibodies than the bulk of the cells in clusters. It is possible that small aggregates of cells migrate away from the large clusters, or that small groups of cells adhere to the larger clusters: the present data can not distinguish which process occurs. However, most of the cells migrating towards the medullary region were in the form of small NCAM-positive L1-negative groups, which was confirmed by cell counting (see below). Double labelling for PNMT and L1 also showed that the PNMT-negative cells in the clusters corresponded to the L1-positive cells ob-

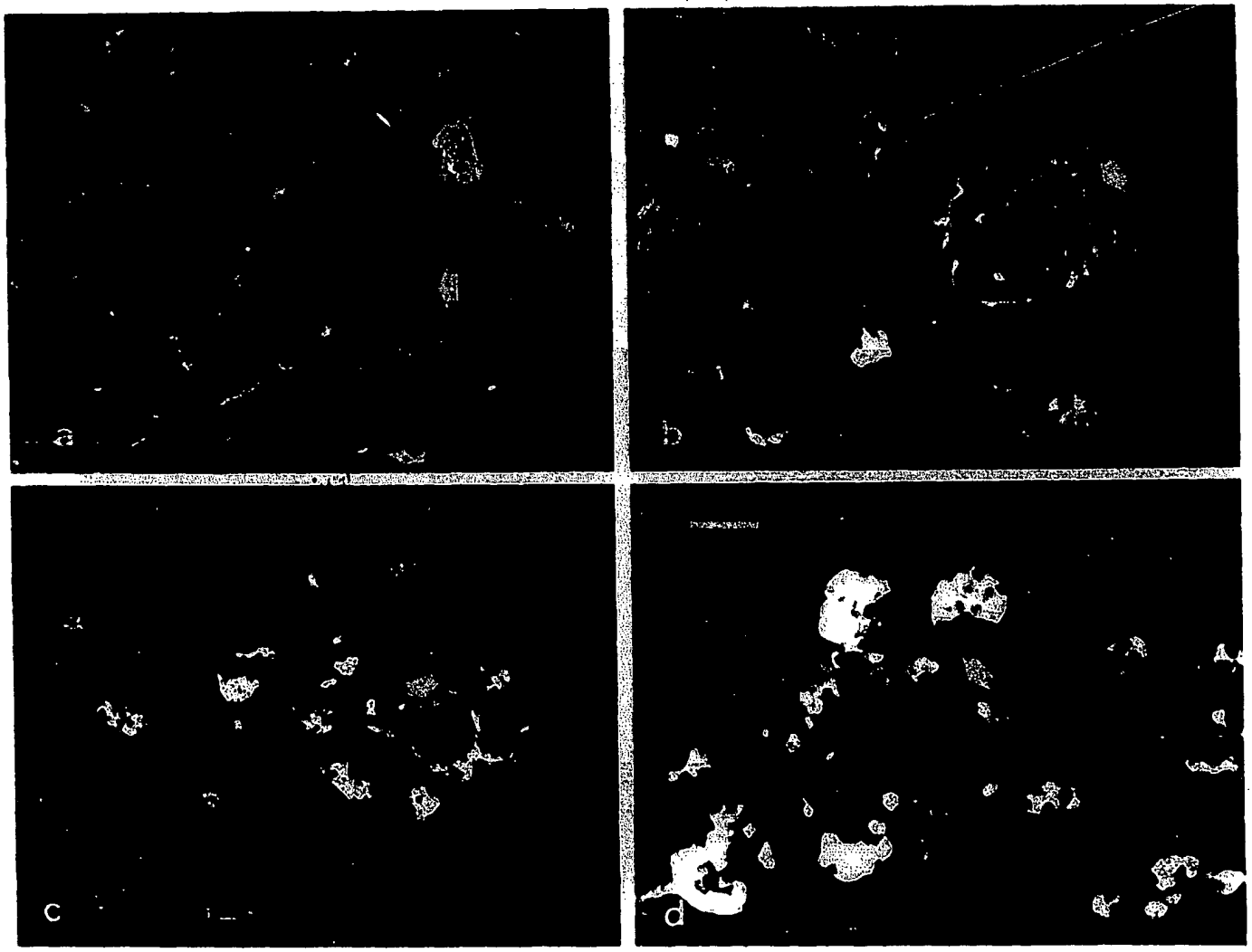

Fig. 2. Immunofluorescent stuining of adrenal gland for NCAM (a,b) and TH (c,d) at E18. Stained cells are dispersed in either small groups or large clusters (arrows). Cells in the large clusters stain more weakly for TH than those in small groups. Bar = 40 μm.

Fig. 4. Electron micrograph of L1 immunolabelled adrenal cortex from E18 rat, demonstrating the pronounced surface staining of unmyelinated nerve fibres (nf) traversing unstained cortex. Section not counterstained. Bar = 1 μm.

Golgi apparatus, abundant mitochondria, many polyribosomes and a well developed reticulum, but few secretory granules were observed.

20th embryonic day

The immunofluorescent staining pattern at E20 was similar to that observed at E18 except that more chromoblasts were present within the gland. The two distinct types of group noted at earlier stages could be easily distinguished by their size and staining characteristics. Although these were concentrated mostly in the central region, neither small nor large groups were compacted together at this stage. The difference between TH staining intensity of the L1-positive, PNMT-negative cells in large clusters and that of cells in small groups was still marked.

Postnatal day 0

At early embryonic ages groups of future chromaffin cells are relatively widely dispersed in the cortical primordium, but a progressive compaction occurs between 2 days before birth and 4 days after birth. Thus, already at the day of birth, groups of NCAM- and TH-positive chromoblasts were found in contact, apparently forming much larger groups (Fig. 6a,b). Some clusters of L1-reactive cells with lower levels of TH were still observed at this stage (Fig. 6c,d), but in

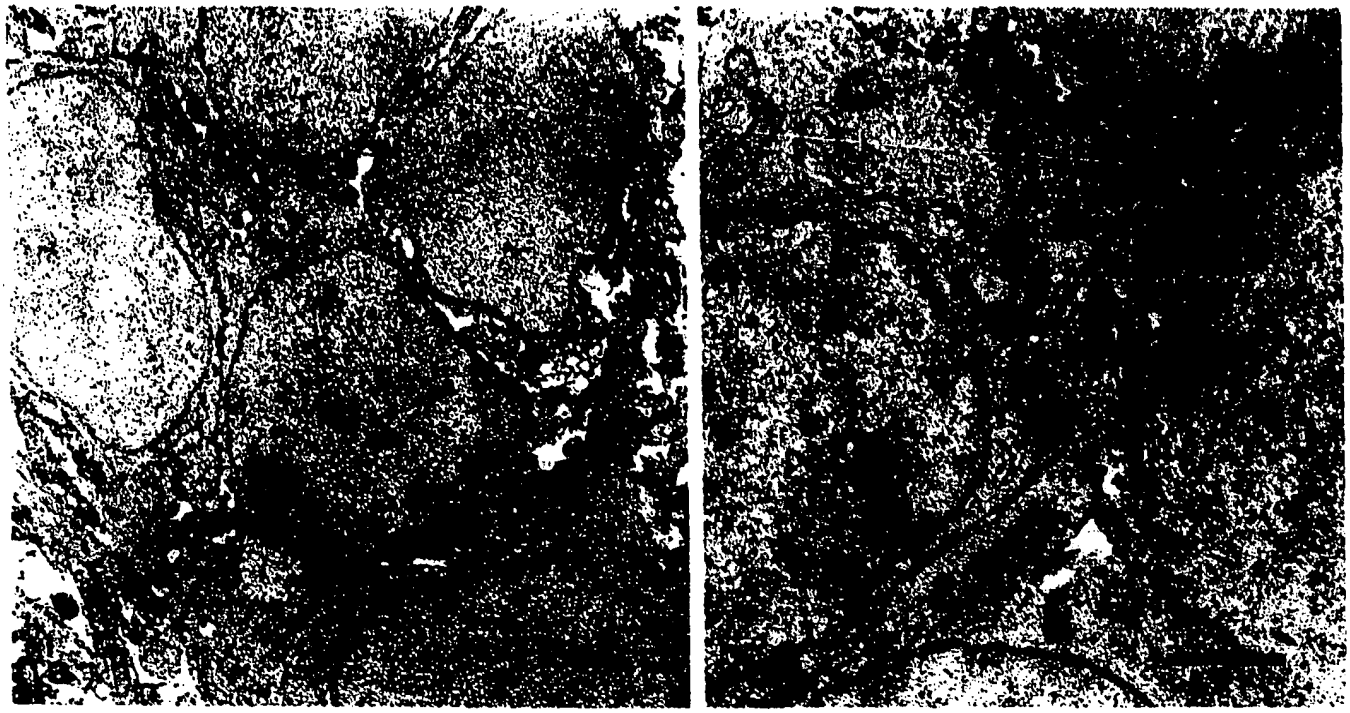

Fig. 5. a: ultrastructural appearance of L1-positive cells in an intra-adrenal cluster at E18. These cells have relatively little cytoplasm, but at higher magnification (b) the abundant mitochondria, well developed reticulum and Golgi apparatus are evident. Section not counterstained. Bars = 1 μm.

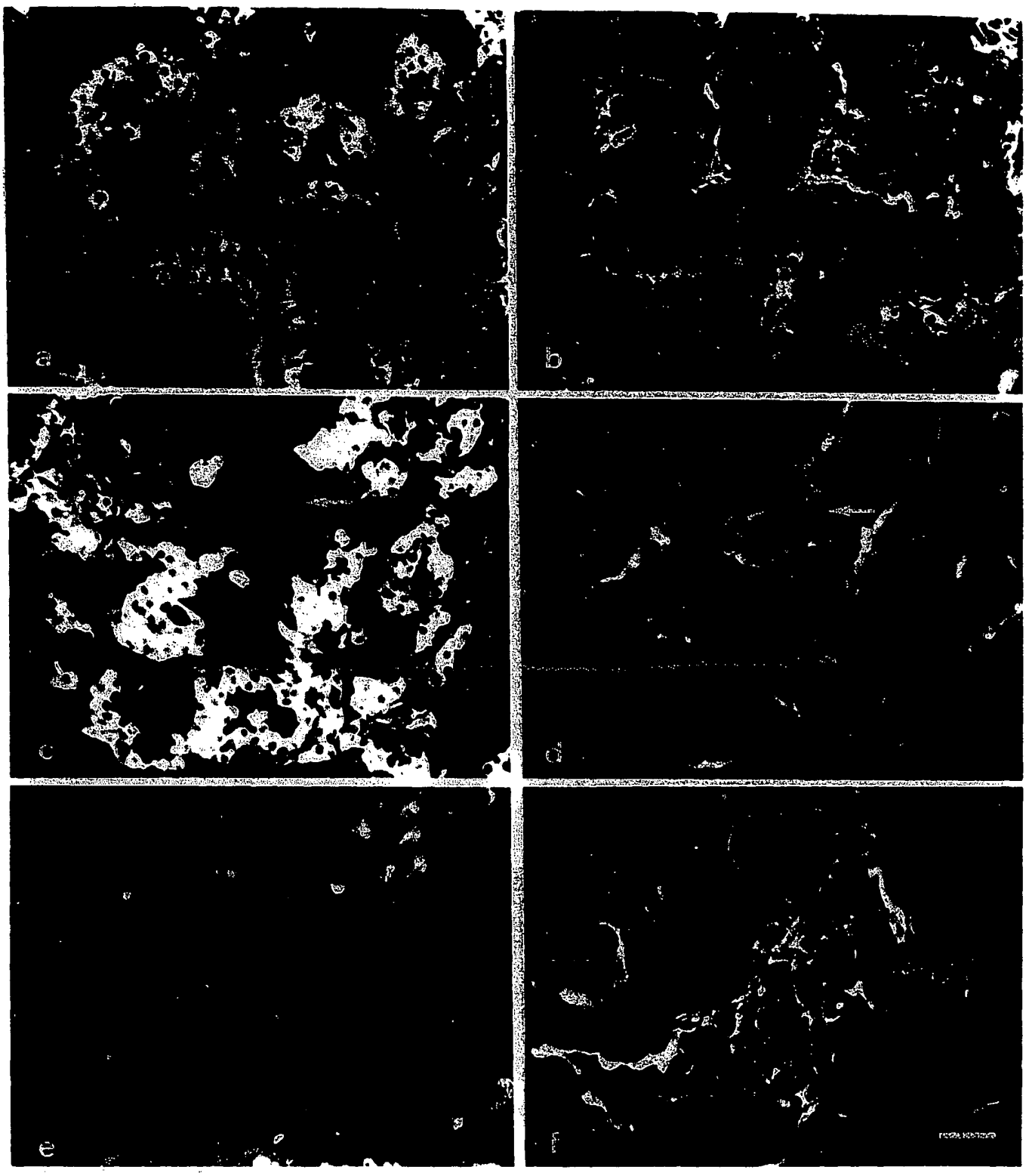

Fig. 6. Double immunofluorescence labelling of intra-adrenal cells at P0 for TH, NCAM (a,b); TH, L1 (c,d); PNMT, L1 (e,f). Note that all TH-positive cells are aso positive for NCAM. Clusters of L1-positive cells which are less intensely positive for TH persist (arrows), although smaller groups of L1-positive cells with higher levels of TH are also seen (small arrows). L1-positive cells remain PNMT-negative (e,f). Bar = 40 μm.

general these were smaller than at earlier stages. Small groups of L1-positive cells which are more reactive for TH were also observed. PNMT reactivity was more intense than at E18, but some chromoblasts remained PNMT-negative (Fig. 6e). Double labelling demonstrated that these cells expressed L1 (Fig. 6f).

Postnatal days 4–14

The segregation between the adrenal medulla and the surrounding cortex is much more distinct during the first two postnatal weeks compared to embryonic stages. NCAM staining persists in all developing medullary chromaffin cells during this period (Fig. 7a,b). Throughout embryonic and early postnatal development, antibodies against NCAM also reacted with the outermost cell layer of the adrenal cortex, while the rest of the cortex remained unlabelled (Fig. 7c), but the cortical periphery was never found to be labelled for L1. The isolated large clusters of L1-immunoreactive cells which are less immunoreactive for TH, still discernable up until P0 were more rarely encountered at 4, and 7 days of postnatal life: at these stages L1-positive cells were principally distributed in smaller cell groups surrounded by L1-negative chromaffin cell groups (Fig. 8a,b). Intensity of TH immunoreactivity was found to vary between groups of cells, but no correlation between L1 immunoreactivity and weakness of TH immunofluorescence was found. L1-reactive cells were found to be PNMT-negative. The rare sympathetic neurones also seen in the adrenal medulla

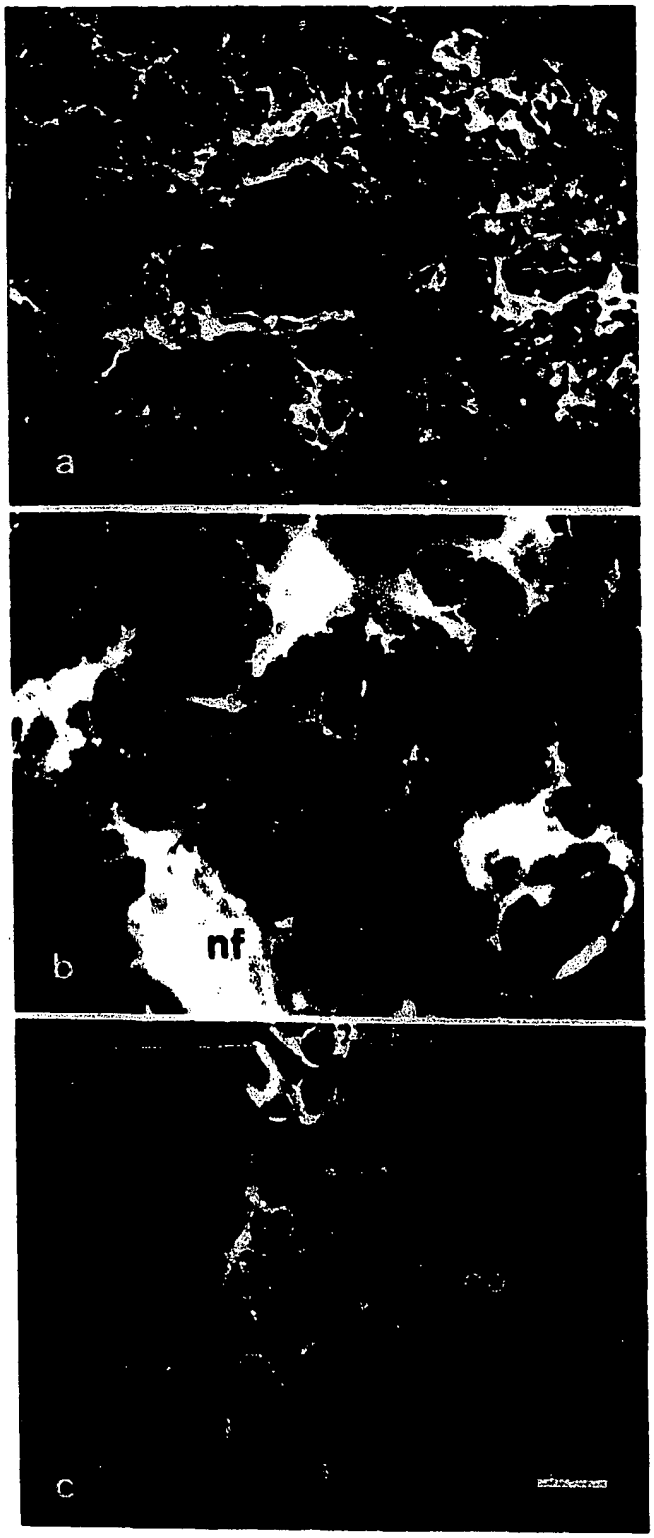

Fig. 7. NCAM immunofluorescence labelling of adrenal medulla at P4 (a) and P7 (b,c). Note the intense reaction of all medullary chromaffin cells and nerve fibres (nf, a, b) and the persistent staining of cells in the peripheral region of the adrenal cortex (co, c). Bar = 40 μm for (a) and 15 μm for (b) and (c).

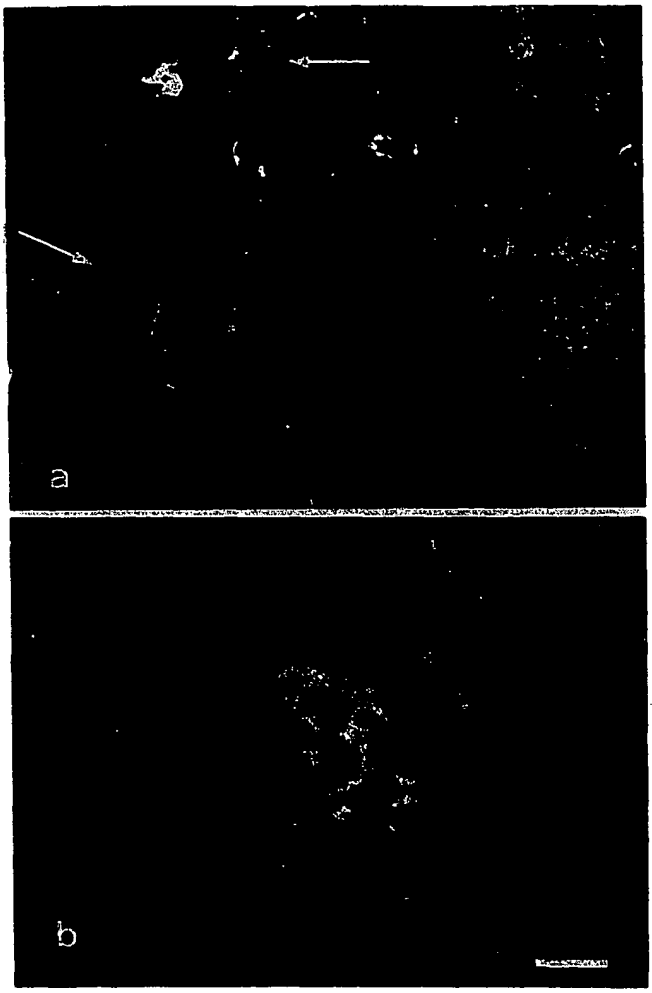

Fig. 8. L1 staining at P4 (a) and P7 (b) is characterised by the presence of small isolated groups of surface labelled cells (arrows in a). Bar = 40 μm for (a) and 25 μm for (b).

TABLE I

L1-positive and weakly TH-positive cells as percentages of total TH-positive cells

Values are means ± S.D. from at least six sections double-labelled for L1 and TH at each age.

Age	*E18*	*E20*	*P0*	*P7*
% L1+cells	24.8±3.2	25.2±5.3	18.1±2.5	17.6±2.1
% weakly TH+cells	25.3±4.4	25.9±5.0	9.8±2.3	4.5±2.5

of the adult rat could be easily identified from P7. These were L1 surface-positive, PNMT-negative, and reacted less intensely for TH than chromaffin cells. Although the size of the adrenal gland increases with age, both the overall tissue structure and the immunostaining pattern obtained during this period with antibodies against the adhesion molecules and the enzymes of catecholamine metabolism is very similar to that of the adult.

Morphometric analysis during development

Estimation of total numbers of different cell types at early ages is rendered difficult by the lack of compactness of the medulla, the non-random distribution of L1-positive cells and the consequent wide variations between sections cut at different levels; some sections contain no L1-reactive chromoblasts. However, the percentages of both L1-positive cells and that of weakly TH-positive cells in terms of the total number of intra-adrenal TH-positive cells could be determined at different ages (Table I). Since we found in separate experiments that all intra-adrenal chromoblasts which reacted either weakly or strongly for TH were also NCAM-positive, the values for L1-positive cells also indicate the percentage of chromoblasts co-expressing both adhesion molecules. This morphometric analysis showed that up until E20 the weakly TH-positive cells, representing about a quarter of all intra-adrenal chromoblasts, corresponded to the subpopulation present in clusters that reacted with anti-L1 antibodies. By birth and during early postnatal development, increasing numbers of L1-positive cells with levels of TH comparable to those in the majority of chromaffin cells were observed (8 and 13% at PO and P7 respectively). In parallel the percentage of weakly TH-positive cells declined during this period to less than one fifth of those present at E18. The percentage of L1-reactive chromoblasts appeared to remain constant between E18 and E20, but declined by approximately 30% between E20 and P0 and then remained constant at approximately 18%.

DISCUSSION

All neural crest precursor cells of the sympathoadrenal cell lineage are subject, during migration to their future sites, to external influences that are considered to determine the nature of the phenotype they express in the adult animal[5,33,63]. Previous data have demonstrated that on arrival in the proximity of the adrenal cortical primordium future chromaffin cells already possess certain noradrenergic cell markers such as TH and dopamine β-hydroxylase[10,60,64,65]. The present results confirm these reports. Other data suggest that in vivo they also express certain characteristics more typical of neurones at this stage, which are subsequently suppressed during normal embryonic development[6,7,66], after cells have migrated into the medullary region of the adrenal gland and are subjected to the PNMT-inductive influence of glucocorticoids secreted by the cortical cells[22,56,62].

The present study set out to compare the expression of two neural cell adhesion molecules by chromaffin cells during adrenal gland development in vivo with that of "marker" enzymes of catecholamine metabolism, in particular TH and PNMT, used as indices of the state of cell differentiation. It has been suggested that by E18 PNMT is present in all medullary chromaffin cells, constituting a "mixed adrenergic/noradrenergic cell type", and that it is subsequently lost from a proportion of cells that are destined to express a noradrenergic phenotype in the adult[65]. The results presented here do not support such a conclusion, but suggest that the induction of PNMT is essentially repressed from the outset in a proportion of chromoblasts, which subsequently develop into the noradrenergic cells of the adult gland. Clusters of PNMT-negative chromoblasts, which are less intensely immunofluorescent than other chromoblasts for TH, have been previously observed in the medullary zone of rat adrenals throughout early development[27]. The present data not only confirm the presence of clusters of such cells in the cortical primordium as early as E16 and that these cells are PNMT-negative, but demonstrate that these cell clusters express the adhesion molecule L1. Such clusters are progressively replaced from E20 and during the 1st week of postnatal life giving rise to smaller groups of L1-positive chromoblasts.Thus those immature cells with a noradrenergic phenotype, as defined by the absence of PNMT and the presence of TH, express L1 from E16, like their adult counterparts[40].

In the central nervous system, L1 is thought to be exclusively expressed by (i.e. represents a marker protein for) neurones[11,20,28,29,43] and we have shown else-

where that L1 is less typical of neuroendocrine cells than neurones[36]. Its synthesis is induced when pheochromocytes transdifferentiate and acquire a neuronal phenotype under the influence of NGF[25,55]. Thus the cells in large clusters which do not express PNMT but are L1-positive and weakly TH-positive may be considered to express a more neuronal than endocrine phenotype. TH levels in sympathetic neurones are lower than those generally found in chromaffin cells[59] and so the weaker TH-immunoreactivity of L1-positive cells correlates with a more typically neuronal phenotype. Groups of cells, morphologically similar to those described here and with properties more characteristic of neurones, have also been reported to be present in both developing human adrenals[19] and embryonic rat adrenals[7]. Similar cells, found to persist in the rat adrenal after birth, were classified as late primitive sympathetic cells or early pheochromoblasts in previous ultrastructural studies[17,18]. In addition, an early neuronal surface marker, B2, characteristic of sympathetic ganglia has been found to be expressed by cells which also express the chromaffin cell characteristic antigen SA1 antigen and low levels of TH, both in extra-adrenal ganglionic complexes and within embryonic adrenal glands[8]: they also probably correspond to the L1-positive cells observed here. Thus the present data accord with earlier suggestions[7,8] that some of the bipotential immature cells originating from the neural crest express a more neuronal phenotype, not only on or shortly after their arrival in the proximity of the adrenal gland, but also well after migration into the cortical primordium.

Coupland[16] suggested many years ago that such intra-adrenal cells were the precursors of both chromaffin and ganglionic lineages. However, to explain the apparent loss of neuronal characteristics in intra-adrenal cells, it has more recently been proposed that most of these cells subsequently die[7,27], since they are situated in an environment unfavourable for their maturation. This seems unlikely in the light of the present observations for two principal reasons. Firstly, such cell clusters appear to be innervated by strongly NCAM- and L1-immunoreactive nerve fibres, although it is improbable that this innervation is physiologically functional at this early stage. Secondly, L1-positive cell groups are observed at all developmental stages, even though L1-positive, weakly TH-positive cell clusters appear to be replaced by smaller groups of L1-positive cells with higher levels of TH at later times. While L1 continues to be expressed by, and thus serves as a marker for, this subset of chromoblasts throughout embryonic and postnatal life, other typically neuronal markers such as PGP9.5, neurofilaments, B2 and SCG-10 appear to be down-regulated[7,28,32]. At E18 the relative immaturity of these cells is demonstrated by their lack of granules and undetectable levels of the secretory granule marker, chromagranin A (our unpublished data), but their maturation into functionally active neuroendocrine cells is suggested by the secretory granules observed later during development in L1-positive cells (our unpublished data). The quantitative data show a decline in the percentage of L1-positive chromoblasts between E20 and P0. This could be due to the migration of cells predominantly destined not to express L1 during this period, to a greater proliferation of such cells or to a partial loss of L1-reactive cells by cell death as proposed by Henion and Landis[27]. The rare sympathetic neurones found in adult glands, which are also L1-positive[40], probably originate from the L1-positive cells observed at early stages, but it may however be concluded that most of the cells survive and differentiate into the noradrenergic cells of the adult gland. Their maturation appears to be slower than the L1-negative chromoblasts, which progress further along the sympathoadrenal pathway towards a more definitive (adrenergic) endocrine phenotype.

Several important questions raised by developmental studies on the adrenal gland remain to be answered. If glucocorticoids secreted by adrenocortical cells are responsible for the induction of PNMT in chromoblasts after their migration into the developing gland, why does a subpopulation remain PNMT-negative and what molecular mechanism is responsible for this? The correlation found between the co-expression of NCAM and L1 and the lack of PNMT in this subpopulation may result from a causal relationship. It is possible that particular interactions between these cells, dependent on the expression of the cell adhesion molecule L1, repress the expression of steroid receptors or inhibit their action. The cytoplasmic tail of the L1 molecule with its associated protein kinase activities[53] could itself be directly involved. These cells would thus be insensitive to the action of corticoids. In support of this hypothesis, it is known that cell interactions can influence neurotransmitter enzyme levels[1-4,32,42]. Further studies are needed to provide insights at the molecular level of such a direct causal relationship between the cellular expression of adhesion molecules and the appearance or maintenance of a given neuroendocrine phenotype.

The present study emphasizes the possible role played by adhesion molecules in constructing the relatively simple tissue structure of this gland. NCAM is expressed very early during the histogenesis of this tissue both by future chromaffin cells in the extra-adrenal blastema and also by cells in the external

layers of the cortical primordium. Since cell adhesion involving NCAM occurs by a homophilic mechanism[23,24], this molecule is probably involved in stabilizing contacts both between arriving cells which accumulate and form the blastema and also between the blastema, as a whole, and the periphery of the adrenal cortex. Subsequently, NCAM appears to be responsible for the continued aggregation of chromoblasts migrating through the cortical primordium in the form of small groups, segregated from NCAM-negative cortical cells. It could also be involved in the nerve/chromaffin cell interaction, since migrating cells even at the earliest times appear to be associated with nerve fibres: cell-substrate adhesion molecules would also be of fundamental importance in the migration of groups of chromoblasts through cortical regions. While it is possible that other as yet undefined adhesion molecules may also be expressed by either adrenergic or noradrenergic cells or both, for example cadherins[21] and NCAM sialylation could influence cell adhesivity at embryonic stages[52], the co-expression of both NCAM and L1 provides an evident molecular basis for segregating the noradrenergic population from adrenergic chromaffin cells by a process of differential adhesion[30]. A consequence of such early cell sorting is that noradrenergic groups would retain their innervation independent of groups of adrenergic cells throughout development. The clusters of NCAM-, L1-positive cells observed at early stages could be initially generated by aggregation of small groups or by proliferation of cells in small groups, since chromoblast multiplication is known to occur during late embryonic and early postnatal life[18]. This is unlikely however, since small isolated groups of L1-positive cells are not observed during early histogenesis. It is thus probable that clusters are preassembled outside the gland and migrate from the extra-adrenal blastema already in this form. It is likely that L1 expression is down-regulated in a large proportion of chromoblasts just before or as they enter the gland.

In summary, the histogenesis of the adrenal medulla appears to be critically dependant on the expression of certain adhesion molecules in a cell phenotype specific manner (Fig. 9). Both NCAM and L1 are present in cells of the extra-adrenal blastema expressing TH. However, while NCAM continues to be expressed in all chromoblasts after their migration into the gland, L1 is found only in a subpopulation, indicating that it is down-regulated in the majority of future chromaffin cells just before their migration into the cortical primordium. Expression of L1 precedes the appearance of PNMT and its continued expression may preclude

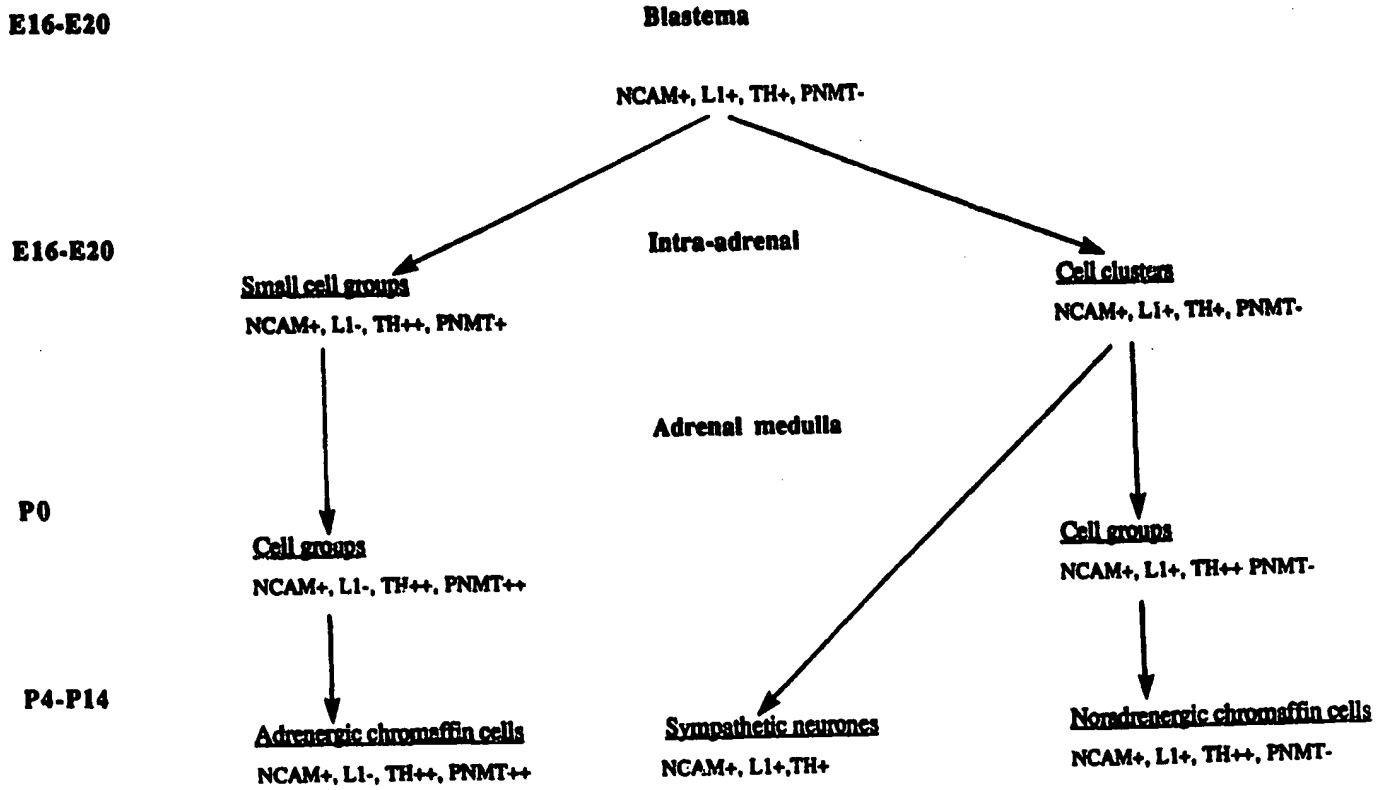

Fig. 9. Summary of CAM and catecholamine metabolic enzyme expression during adrenal gland histogenesis. Throughout development two distinct populations can be distinguished by their expression of L1 or PNMT and, at early stages, by their levels of TH. Dopamine β-hydroxylase is present in chromoblasts at all stages[64] and chromogranin A is progressively induced in intra-adrenal cells of both phenotypes from E16 to P0 (our unpublished results).

PNMT induction in a subpopulation of chromoblasts. The specific cellular and temporal expression of the adhesion molecules NCAM and L1 during embryogenesis are likely to be principal factors which determine the initial attachment of future medullary cells to the cortical periphery, the aggregation of cells during their migratory phase through the cortex, and the stabilization of nerve fibre/chromaffin cell contacts. Finally, this study highlights the potential role of cell adhesion molecules in adrenal gland development in causing both the aggregation of immature cells which are the likely progenitors of adult noradrenergic chromaffin cells and their segregation from the future adrenergic cell population as homophenotypic cell groups.

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REFERENCES

1 Acheson, A.L. and Thoenen, H., Cell contact-mediated regulation of tyrosine hydroxylase synthesis in cultured bovine adrenal chromaffin cells, *J. Cell Biol.*, 97 (1983) 925–928.
2 Acheson, A.L. and Rutishauser, U., Neural cell adhesion molecule regulates cell contact-mediated changes in choline acetyltransferase activity of embryonic chick sympathetic neurons, *J. Cell Biol.*, 106 (1988) 479–486.
3 Adler, J.E. and Black, I.B., Sympathetic neuron density differentially regulates transmitter phenotypic expression, *Proc. Natl. Acad. Sci. USA*, 82 (1985) 4296–4300.
4 Adler, J.E. and Black, I.B., Membrane contact regulates transmitter phenotypic expression, *Dev. Brain Res.*, 30 (1986) 237–241.
5 Anderson, D.J., The neural crest 'I lineage problem: neuropoiesis? *Neuron*, 3 (1989) 1–12.
6 Anderson, D.J. and Axel, R., Molecular probes for the development and plasticity of neural crest derivatives, *Cell*, 42 (1985) 649–662.
7 Anderson, D.J. and Axel, R., A bipotential neuroendocrine precursor whose choice of cell fate is determined by NGF and glucocorticoids, *Cell*, 47 (1986) 1079–1090.
8 Anderson, D.J., Carnahan, J.F., Michelson, A. and Patterson, P.H., Antibody markers identify a common progenitor to sympathetic neurons and chromaffin cells in vivo and reveal the timing of commitment to neuronal differentiation in the sympathoadrenal lineage, *J. Neurosci.*, 11 (1991) 3507–3519.
9 Bock, E., Richter-Landsberg, C., Faissner, A. and Schachner, M., Demonstration of immuno-chemical identity between the nerve growth factor-inducible large external (NILE) glycoprotein and the cell adhesion molecule L1, *EMBO J.*, 4 (1985) 2765–2768.
10 Bohn, M.C., Goldstein, M. and Black, I.B., Role of glucocorticoids in expression of the adrenergic phenotype in rat embryonic adrenal gland, *Dev. Biol.*, 82 (1981) 1–10.
11 Chuong, C.M. and Edelman, G.M., Alterations in neural cell adhesion molecules during development of different regions of the nervous system, *J. Neurosci.*, 4 (1984) 2354–2368.
12 Chuong, C.M, Crossin, K.L. and Edelman, G.M., Sequential expression and differential function of multiple adhesion molecules during the formation of cerebellar cortical layers, *J. Cell Biol.*, 104 (1987) 331–342.
13 Cochard, P. and Paulin, D., Initial expression of neurofilaments and vimentin in the central and peripheral nervous system of the mouse embryo in vivo, *J. Neurosci.*, 4 (1984) 2080–2094.
14 Cochard, P., Goldstein, M. and Black, I.B., Initial development of the noradrenergic phenotype in autonomic neuroblasts of the rat embryo in vivo, *Dev. Biol.*, 71 (1979) 100–114.
15 Cohen, A.M., Factors directing the expression of sympathetic nerve traits in cells of neural crest origin, *J. Exp. Zool.*, 179 (1972) 167–182.
16 Coupland, R.E., *The Natural History of the Chromaffin Cell*, Longmans, London, 1965.
17 Coupland, R.E., The natural history of the chromaffin cell—Twenty-five years on the beginning, *Arch. Histol. Cytol.*, 52 Suppl. (1989) 331–341.
18 Coupland, R.E. and Tomlinson, A., The development and maturation of adrenal medullary chromaffin cells of the rat in vivo: a descriptive and quantitative study, *Int. J. Dev. Neurosci.*, 7 (1989) 419–438.
19 Cooper, M.J., Hutchins, G.M. and Israel, M.A., Histogenesis of the human adrenal medulla: an evaluation of the ontogeny of chromaffin and nonchromaffin lineages, *Am. J. Pathol.*, 137 (1990) 605–615.
20 Daniloff, J.K., Chuong, C.M., Levi, G. and Edelman, G.M., Differential distribution of cell adhesion molecules during histogenesis of the chick nervous system, *J. Neurosci.*, 6 (1986) 739–758.
21 Doherty, P., Ashton, S.V., Moore, S.E. and Walsh, F.S., Morphoregulatory activities of NCAM and N-cadherin can be accounted for by G protein-dependent activation of L- and N-type neuronal Ca^{2+} channels, *Cell*, 57 (1991) 21–33.
22 Doupe, A.J., Landis, S.C. and Patterson, P.H., Environmental influences in the development of neural crest derivatives: glucocorticoids, growth factors, and chromaffin cell plasticity, *J. Neurosci.*, 5 (1985) 2119–2142.
23 Edelman, G.M., Expression of cell adhesion molecules during embryogenesis and regeneration, *Exp. Cell Res.*, 161 (1984) 1–16.
24 Edelman, G.M., Cell surface modulation and marker multiplicity in neural patterning, *Trends in Neurosci.*, 713 (1984) 78–82.
25 Freidlander, D.R., Grumet, M. and Edelman, G.M., Nerve growth factor enhances expression of neuron-glia cell adhesion molecule in PC12 cells, *J. Cell Biol.*, 102 (1986) 413–419.
26 Goridis, C. and Wille, W., The three size classes of mouse NCAM proteins arise from a single gene by a combination of alternative splicing and use of different polyadenylation sites. *Neurochem. Int.*, 12 (1988) 269–272.
27 Henion, P.D. and Landis, S.C., Asynchronous appearance and topographic segregation of neuropeptide-containing cells in the developing rat adrenal medulla, *J. Neurosci.*, 10 (1990) 2886–2896.
28 Hoffman, S., Freidlander, D.R., Chuong, C.M., Grumet, M. and Edelman, G.M., Differential contributions of Ng-CAM and N-CAM to cell adhesion in different neural regions, *J. Cell Biol.*, 103 (1986) 145–158.
29 Joosten, E.A.J. and Gribnau, A.A.M., Immunocytochemical localization of cell adhesion molecule L1 in developing rat pyramidal tract, *Neurosci. Lett.*, 100 (1989) 94–98.
30 Kadmon, G., Kowitz, A., Altvogt, P. and Schachner, M., The neural cell adhesion molecule N-CAM enhances L1-dependent cell–cell interactions, *J. Cell. Biol.*, 110 (1990) 193–208.
31 Kent, C. and Clarke, P.J., The immunolocalisation of the neuroendocrine specific protein PGP9.5 during neurogenesis of the rat, *Dev. Brain Res.*, 58 (1991) 147–150.
32 Kessler, J.A., Conn, G. and Hatcher, V.B., Isolated plasma membranes regulate neurotransmitter expression and facilitate effects of a soluble brain cholinergic factor, *Proc. Natl. Acad. Sci. USA*, 83 (1986) 3528–3532.
33 Landis, S.C. and Patterson, P.H., Neural crest cell lineages, *Trends Neurosci.*, 4 (1981) 172–175.
34 Langley, O.K. and Aunis, D., Ultrastructural demonstration of D2-protein in adrenal medulla, *Cell Tiss. Res.*, 238 (1984) 497–502.
35 Langley, O.K., Aletsee-Ufrecht, M.C., Grant, N.J. and Gratzl, M., Expression of the neural cell adhesion molecule N-CAM in endocrine cells, *J. Histochem. Cytochem.*, 37 (1989) 781–791.
36 Langley, O.K., Aletsee-Ufrecht, M.C. and Gratzl, M., Comparison of the expression of two cell adhesion molecules in endocrine tissues, *J. Neurochem.*, 52 Suppl. (1989) S133A.

37 Langley, K. and Gratzl, M., N-CAM as a marker for neural and endocrine cells. In M. Gratzl and K. Langley (Eds.), *Markers for Neural and Endocrine Cells, Molecular and Cell Biology Diagnostic Applications*, VCH, Weinheim, New York, 1991, pp. 133–178.

38 Le Douarin, N.M. and Teillet, M.A., Experimental analysis of the migration and differentiation of neuroblasts of the autonomic nervous system and of neuroectodermal mesenchymal derivatives, using a biological cell marking technique, *Dev. Biol.*, 41 (1974) 162–184.

39 Le Douarin, N.M., Teillet, M.A. and Le Lievre, C., Influence of the tissue environment on the differentiation of neural crest cells. In J.W. Lash and M.M. Burger (Eds), *Cell and Tissue Interactions*, Raven, New York, 1977, pp.

40 Léon, C., Grant, N.J., Aunis, D. and Langley, K., L1 cell adhesion molecule is expressed by noradrenergic chromaffin cells: a possible major role for L1 in adrenal medullary design, *Eur. J. Neurosci.*, 4 (1992).

41 Lindner, J., Rathjen, F.G. and Schachner, M., L1 mono- and poly-clonal antibodies modify cell migration in early postnatal mouse cerebellum, *Nature*, 305 (1983) 427–429.

42 Lucas, C.A., Edgar, D. and Thoenen, H., Regulation of tyrosine hydroxylase and choline acetyltransferase activities by cell density in the PC12 rat pheochromocytoma clonal cell line, *Exp. Cell Res.*, 121 (1979) 79–86.

43 Miragall, F., Kadmon, G. and Schachner, M., Expression of L1 and N-CAM cell adhesion molecules during development of the mouse olfactory system, *Dev. Biol.*, 135 (1989) 272–286.

44 Moro, M.A., Garcia, A.G. and Langley, O.K., Characterisation of two chromaffin cell populations isolated from bovine adrenal medulla, *J. Neurochem.*, 57 (1991) 363–369.

45 Norr, S., In vitro analysis of sympathetic neuron differentiation from chick neural crest cells, *Dev. Biol.*, 34 (1973) 16–38.

46 Nybroe O., Linneman, D. and Bock, E., NCAM biosynthesis in brain, *Neurochem. Int.*, 12 (1988) 251–262.

47 Piggot, R. and Kelly, J.S., Immunoctochemical and biochemical studies with the monoclonal antibody 69 A1: similarities of the antigen with cell adhesion molecules L1, NILE and Ng-CAM. *Dev. Brain Res.*, 29 (1986) 111–122.

48 Pollerberg, E., Burridge, K., Krebs, K., Goodman, S. and Schachner, M., The 180 kD component of the neural cell adhesion molecule N-CAM is involved in cell–cell contacts and cytoskeleton-membrane interactions, *Cell Tiss. Res.*, 250 (1987) 227–236.

49 Rathjen, F.G., A neurite outgrowth-promoting molecule in developing fiber tracts, *Trends Neurosci.*, 11 (1988) 183–184.

50 Rathjen, F.G., Wolff, J.M., Frank, R., Bonhoeffer, F. and Rutishauser, U., Membrane glycoproteins involved in neurite fasciculation, *J. Cell. Biol.*, 104 (1987) 1987, 343–353.

51 Rutishauser, U., Developmental biology of a neural cell adhesion molecule, *Nature*, 310 (1984) 549–554.

52 Rutishauser, U., Acheson, A., Hall, A.K., Mann, D.M. and Sunshine, J., The neural cell adhesion molecule (NCAM) as a regulator of cell–cell interactions, *Science*, 240 (1988) 53–57.

53 Sadoul, R. Kirchhoff, F. and Schachner, M., A protein kinase activity is associated with and specifically phosphorylates the neural cell adhesion molecule L1, *J. Neurochem.*, 53 (1989) 1471–1478.

54 Sajovic, P., Kouvelas, E. and Trenker, E., Probable identity of NILE glycoprotein and the high molecular weight component of L1 antigen, *J. Neurochem.*, 47 (1986) 541–546.

55 Salton, S.R.J., Richter-Landsberg, C., Green, L.A. and Shelanski, M.L., Nerve growth factor inducible large external (NILE) glycoprotein: studies of a central and peripheral neuronal marker. *J. Neurosci.*, 3 (1983) 441–454.

56 Seidl, K. and Unsicker, K., The determination of the adrenal medullary cell fate during embryogenesis, *Dev. Biol.*, 136 (1989) 481–490.

57 Stallcup, W.B. and Beasley, L., Involvement of the nerve growth factor-inducible large external glycoprotein (NILE) in neurite fasciculation in primary cultures of rat brain, *Proc. Natl. Acad. Sci. USA*, 82 (1985) 1276–1280.

58 Stallcup, W.B., Beasley, L. and Levine, J.M., Antibody against nerve growth factor-inducible large external (NILE) glycoprotein labels nerve fiber tracts in the developing rat nervous system, *J. Neurosci.*, 5 (1985) 1090–1101.

59 Stein, R., Orit, S. and Anderson, D.J., The induction of a neural-specific gene, SGC10, by nerve growth factor in PC12 cells is transcriptional, protein synthesis dependant, and glucocorticoid inhibitable, *Dev. Biol.*, 127 (1988) 316–325.

60 Teitelman, G., Baker, H., Joh, T.H. and Reis, D.J., Appearance of catecholamine-synthesizing enzymes during development of rat sympathetic nervous system: possible role of tissue environment, *Proc. Natl. Acad. Sci. USA*, 76 (1979) 509–513.

61 Thiery, J.-P., Duband, J.-L., Rutishauser, U. and Edelman, G.M., Cell adhesion molecules in early chicken embryogenesis, *Proc. Natl. Acad. Sci. USA*, 79 (1982) 6737–6741.

62 Unsicker, K., Krisch, B., Otten, U. and Thoenen, H., Nerve growth factor-induced fiber outgrowth from isolated rat adrenal chromaffin cells: impairment by glucocorticoids, *Proc. Natl. Acad. Sci. USA*, 75 (1978) 3498–3502.

63 Unsicker, K., Seidl, K. and Hofman, H.D., The neuro-endocrine ambiguity of sympathoadrenal cells, *Int. J. Dev. Neurosci.*, 7 (1989) 413–417.

64 Verhofstad, A.A.J., Hökfelt, T., Goldstein, M., Steinbusch, H.W.M. and Joosten, H.W.J., Appearance of tyrosine hydroxylase, aromatic amino-acid decarboxylase, dopamine β hydroxylase and phenylethanolamine *N*-methyltransferase during the ontogenesis of the adrenal medulla, *Cell Tiss. Res.*, 200 (1979) 1–13.

65 Verhofstad, A.A.J., Coupland, R.E., Parker, T.R. and Goldstein, M., Immunohistochemical and biochemical study on the development of the noradrenaline- and adrenaline-storing cells of the adrenal medulla of the rat, *Cell Tiss. Res.*, 242 (1985) 233–243.

66 Vogel K.S. and Weston, J.A., The sympathetic lineage in avian embryos. 1. Adrenal cells loose neuronal traits during embryogenesis, *Dev. Biol.*, 139 (1990) 1–12.